Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes

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Abstract
A study was undertaken to evaluate the impact of the application of several fungicide treatments used in Spanish vines on *Aspergillus carbonarius* growth and ochratoxin A production. Three trials were designed in order: (1) to screen 26 fungicides at the doses recommended by manufacturers on grape-like synthetic medium at 20 and 30°C; (2) to find out the minimum inhibitory concentration of each fungicide for *A. carbonarius* growth on synthetic medium; and (3) to investigate the effect of several fungicides on *A. carbonarius*-inoculated grapes. In synthetic medium nine fungicides significantly reduced *A. carbonarius* growth rate. Meanwhile, 13 fungicides completely inhibited its growth. In general, growth was faster at 30°C than at 20°C, contrary to ochratoxin A production. Fungicides that stopped fungal growth also inhibited ochratoxin A production, but not all the fungicides that reduced growth reduced the ochratoxin A synthesis. In general, fungicides that contained copper or strobilurins reduced both growth and ochratoxin A production, contrary to sulphur fungicides. At the optimum temperature for *A. carbonarius* growth of 30°C, higher amounts of fungicide were needed to prevent fungal growth than at 20°C. Among the fungicides that inhibited *A. carbonarius* growth on synthetic medium at the initial doses, cyprodinil seemed to be the active ingredient more effective at stopping fungal growth when testing reduced doses. The fungicide effect on grapes was similar to that on synthetic medium. Both infection and ochratoxin A production were reduced when using cyprodinil (37.5%) plus fludioxonil (25%) and azoxystrobin (25%). Penconazole (10%) also showed a clear reduction in ochratoxin A production at both temperatures, although infection was only reduced at 20°C. Ochratoxin A reduction was strain and temperature-dependent. In general, fenhexamid (50%), mancozeb (80%) and copper hydroxide (80%) plus copper (50%) enhanced infection and ochratoxin A production.

Keywords: Fungicides, *Aspergillus carbonarius*, ochratoxin A, grapes

Introduction
Ochratoxins are fungal secondary metabolites produced mainly by fungi from the genera *Aspergillus* and *Penicillium*, which are present in a wide variety of foods. Ochratoxin A (OTA) is one of the more studied mycotoxins in wines nowadays, being recently regulated in the European Union (European Commission 2005), mainly due to its high toxicity and presence in wines all over the world. Its production in grapes from the Mediterranean area is associated with different *Aspergillus* spp., mostly black aspergilli and, among them, *A. carbonarius* (Cabañes et al. 2001; Battilani et al. 2003; Belli et al. 2004a).

Prevention of the growth of mycotoxin-producing fungi is the most effective strategy for controlling the presence of mycotoxins in foods. This could be achieved by knowing the critical limits of different eco-physiological factors affecting fungal infection and mycotoxin synthesis, but in many cases the use of fungicides is the only efficient, cost-effective and often successful way to prevent mould growth (Munimbazi et al. 1997).

The aim of the present study was to evaluate the impact of the application of several fungicides to grapes, on *A. carbonarius* growth and OTA production. The experimental design was divided in three parts in order: (1) to screen the main fungicides used
in Spanish vines to test their efficiency against *A. carbonarius* growth and OTA production on synthetic nutrient medium (SNM); (2) to find out the minimum inhibitory concentration (MIC) of each fungicide for *A. carbonarius* growth on SNM; and (3) to investigate the effect of several fungicides on growth and OTA production of *A. carbonarius* inoculated on grapes.

**Materials and methods**

**Screening of the main fungicides used in vines on synthetic grape-like medium**

Three ochratoxigenic *A. carbonarius* strains (3.161, 3.162 and 3.168, grape-isolated from Italy, France and Spain, respectively) were used to inoculate centrally (10⁶ spores ml⁻¹) Petri dishes containing 20 ml of synthetic nutrient medium (SNM), which had a composition similar to grapes, and a water activity (a_w) level of 0.99 (Bellı́ et al. 2004b). Strains were held in the culture collection of the Food Technology Department, University of Lleida, Spain.

Twenty-six fungicides commonly used on Spanish vines were added to the medium at the doses recommended by manufacturers (Table I). Each fungicide was aseptically added to the autoclaved medium before it was plated. No fungicides were added to the control plates. Plates were incubated at 20 and 30°C inside plastic bags. Colony diameters were measured after 3, 5 and 7 days and OTA was extracted after 7 days following the method of Bragulat et al. (2001).

High-performance liquid chromatography (HPLC) with fluorescence detection (Waters 474, Milford, MA, USA) (λ_exc = 330 nm; λ_em = 460 nm) was used for OTA analysis. The mobile phase was acetonitrile–water–acetic acid (57:41:2) (1.0 ml min⁻¹) and a C₁₈ column (Waters Spherisorb 5 μm, ODS2, 4.6 × 250 mm) was used. The injection volume and retention time were 25 μl and 7.1 min, respectively. The detection limit of the analysis was 0.02 μg OTA g⁻¹ of SNM, based on a signal-to-noise ratio of 3:1. The OTA standard was from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). The standard solution was made in methanol and concentration confirmed by using an ultraviolet light spectrophotometer. Three repetitions were carried out for both growth and OTA studies.

**Minimum doses preventing Aspergillus carbonarius growth on synthetic grape-like medium**

All the fungicides that prevented *A. carbonarius* growth at the doses recommended by the manufacturers in the previous experiment (n = 13) were selected for this study in order to find the minimum

| Code | Fungicide | Company | Composition | Dose |
|------|-----------|---------|-------------|------|
| F1   | TOPAS     | Syngenta| Penconazole 10% p/v | 0.35 ml⁻¹ |
| F2   | SHIRLAN 500SC | Syngenta | Fluazinam 50% p/v | 2 ml⁻¹ |
| F3   | QUADRIS   | Syngenta| Afoxystrobin 25% p/v | 2.25 ml⁻¹ |
| F4   | Experimental product | Syngenta | CGA302130 | 2 ml⁻¹ |
| F5   | CUPROCOL  | Syngenta| Copper oxychloride 70% p/v | 2 ml⁻¹ |
| F6   | GEOXE     | Syngenta| Fludioxonil 50% | 0.5 g⁻¹ |
| F7   | Experimental product | Syngenta | CGA379438 | 1 g⁻¹ |
| F8   | THIOVT JET | Syngenta | Sulfur 80% WG | 4 g⁻¹ |
| F9   | SWITCH    | Syngenta| Cypromidil 37.5% plus fludioxonil 25% | 1.8 g⁻¹ |
| F10  | CHORUS 50 WG | Syngenta | Cypromidil 50% | 2 g⁻¹ |
| F11  | SUMISCLEX 50WP | Masso | Procyxadine | 1 g⁻¹ |
| F12  | RIDOMIL GOLD COMBI | Syngenta | Folpet 40% plus mefenoxam 5% WP | 2 g⁻¹ |
| F13  | QUADRIS DUO | Syngenta | Afoxystrobin 18.7% plus cydocyanil 12% WG | 2.25 g⁻¹ |
| F14  | TELDOR    | Bayer   | Fenhexamid 50% p/p | 2 g⁻¹ |
| F15  | EUPAREN M | Bayer   | Tolyfluanid 50% p/p | 1.75 g⁻¹ |
| F16  | FOLICUR 25EW | Bayer | Tebuconazole 25% p/v | 0.70 ml⁻¹ |
| F17  | FLINT     | Bayer   | Trifloxystrobin 50% p/p | 0.13 g⁻¹ |
| F18  | CAPLUQ-50 | Luqsa   | Captan 50% p/p | 3.5 g⁻¹ |
| F19  | CARBENLUQ-50 | Luqsa | Carbendazim 50% p/p | 0.6 g⁻¹ |
| F20  | COBRELUQ-50 | Luqsa | Copper oxychloride 50% p/p | 3.5 g⁻¹ |
| F21  | CUPROLUQ  | Luqsa   | Cuprous oxide 75% p/p | 2 g⁻¹ |
| F22  | LUQSARZUFRE | Luqsa | Sulfur 80% p/p | 5 g⁻¹ |
| F23  | MANCOZEB 80 | Luqsa | Mancozeb 80% p/p | 3 g⁻¹ |
| F24  | TMTD 80   | Luqsa   | Tiram 80% p/p | 2.5 g⁻¹ |
| F25  | ZICOLUQ 320 | Luqsa | Copper oxychloride 22% p/p plus mancozeb 17.5% p/p | 5 g⁻¹ |
| F26  | HIDROXILUQ 800 | Luqsa | Copper hydroxide 80% p/p plus copper 50% p/p | 2 g⁻¹ |
fungicide. For culturing, 10³ spore ml⁻¹ (90–100%) throughout the experiment. In control onto a grid inside plastic boxes containing 300 ml was aseptically removed. Afterwards, 20 grapes were dipped in a fungicide solution for 30 s and placed was aseptically removed. Afterwards, 20 grapes were surface disinfected by (Red Globe variety) were surface disinfected by and used to obtain the growth rate under each concentration that inhibited the growth of this mould (MIC). Spore suspensions of 3.162 and 3.168 A. carbonarius strains were adjusted to contain approximately 10⁶ spores ml⁻¹ for use as inoculum. SNM plates (0.99a₀) with decreasing concentrations of those fungicides (D, dose recommended by the manufacturer; d₁, 0.75 × D; d₂, 0.5 × D; d₃, 0.25 × D; d₄, 0.1 × D; d₅, 0.01 × D; and d₆, 0.005 × D) were single-point inoculated and incubated at 20 and 30°C. Growth was measured daily over 30 days. Three repetitions were carried out.

Effect of fungicides on grapes

The effect of six fungicides applied directly to grapes was investigated. F3, F14 and F26 were used at the dose recommended by the manufacturer, and F1, F9 and F23 were used at d₄ (0.1 × D). Table grapes (Red Globe variety) were surface disinfected by dipping them in NaClO (0.1% Cl) and ethanol (70%) solutions for 30 s and an excess of moisture was aseptically removed. Afterwards, 20 grapes were dipped in a fungicide solution for 30 s and placed onto a grid inside plastic boxes containing 300 ml of water to keep a high relative humidity rate (90–100%) throughout the experiment. In control treatments, grapes were dipped in water instead of fungicide. For culturing, 10³ spore ml⁻¹ suspensions of two A. carbonarius strains (3.162 and 3.168) were sprayed onto the grapes. After 7 days of incubation at 20 and 30°C, the percentage of grapes infected by A. carbonarius was assessed. The whole set of grapes of each treatment were crushed, and filtered through Whattman No. 1 filter paper under vacuum. OTA was extracted from this must following the method of Bezzo et al. (2000). A total of 25 μl of each sample was injected into the HPLC system equipped with a fluorescence detector (Waters 474) (λₑₓcₙ = 230 nm; λₑₘₐₓ = 458 nm) and a C₁₈ column (Waters Spherisorb 5 μm, ODS2, 4.6 × 250 mm). The analysis was performed under isocratic conditions, with acetonitrile 48% and sodium acetate 4 mM/acetic acid (19/1) 52% as the mobile phase, pumped at a flow rate of 1 ml min⁻¹. The injection volume and retention time were 25 μl and 12 min, respectively. The limit of detection of the analysis was 0.05 μg l⁻¹, based on a signal-to-noise ratio of 3:1. OTA was quantified by the external standard method. The ochratoxin standard was from Aspergillus ochraceus (Sigma-Aldrich, Steinheim, Germany). The standard solution was made in methanol and confirmed by using an ultraviolet light spectrophotometer.

Statistical treatment of the results

The regression lines of colony diameters against days after inoculation were calculated for each fungicide and used to obtain the growth rate under each treatment conditions. Fungicide effect on mycelial growth on SNM and OTA production, both in medium and in natural grapes, were analysed statistically with SAS Enterprise Guide software (version 2.0; SAS Institute, Inc., Cary, NC, USA) by analysis of variance followed by either LSMEAN or Duncan multiple range tests. Statistical significance was judged at p < 0.001.

Results

Screening of the main fungicides used in vines on synthetic grape-like medium

Significant differences were detected for the single factors temperature and fungicide and their interaction, while all the strains showed statistically similar growth, regardless of the assayed levels of the remaining factors. Growth was faster at 30°C than at 20°C, except for F2 and F11. Nine fungicides significantly reduced A. carbonarius growth rate at both temperatures (F2, F5, F7, F11, F13, F14, F20, F21 and F26) in comparison with the control treatment, meanwhile 13 fungicides completely inhibited fungal growth at the dose assayed (F1, F4, F6, F9, F10, F12, F15, F16, F18, F19, F23, F24 and F25) (Table II). No significant effects were observed for the remaining four fungicides on growth.

The fungicides had a significant effect on OTA production by A. carbonarius, because obviously the 13 fungicides that prevented fungal growth also inhibited OTA production. Among the remaining fungicides, analysis of variance showed that none of them reduced significantly OTA production. No significant differences were found among the isolates tested, in their response either to temperature or to fungicide treatments. The interaction fungicide × temperature was also significant. Contrary to the growth pattern, OTA production was in general higher at 20°C than at 30°C. Although not having a significant weight, general trends can be drawn from the results. Mean levels of OTA production showed that most of the fungicides that reduced A. carbonarius growth also reduced OTA production, with the exception of F2, F5 and F11, which favoured toxin production at both temperatures. OTA was also favoured by the addition of F7 at 20°C, and F14, F21 and F26 at 30°C. OTA was also higher than the control under the effect of F8 at both temperatures, although growth was only stimulated at 30°C. Contrarily, F17 reduced OTA production, although growth was favoured at 20°C. OTA was favoured at 20 and 30°C under the effect of F22, the unique fungicide that increased fungal growth at both temperatures. Figure 1 compares the growth rate and amount of OTA detected at both
temperatures after the application of each fungicide with the control treatment, which is represented at the origin of coordinates. Control growth and OTA production detected after the application of each fungicide has been subtracted from growth and OTA production by the control treatment. Thus, fungicides in the third quadrant of the graphic resulted in production by the control treatment. Thus, fungi-fungicide has been subtracted from growth and OTA production detected after the application of each fungicide at the origin of coordinates. Control growth and OTA production with the control treatment, which is represented at temperatures after the application of each fungicide.

One-tenth of D (d4), was the MIC of fungicides F4 and captan 50% (F18) at 20°C, and fludioxonil (F6) and tolyfluanid (F15) at both temperatures. A mixture of cyprodinil 37.5% and fludioxonil 25% (F9) and cyprodinil alone (F10) were the most effective fungicides as they hinder growth at the minimum doses assayed (d5 and d6, respectively).

**Effect of fungicides on grapes**

Three fungicides that completely inhibited *A. carbonarius* on SNM: penconazole 10% (F1), cyprodinil 37.5% plus fludioxonil 25% (F9) and mancozeb 80% (F23), and two fungicides that reduced its growth at the initial doses assayed: fenhexamid 50% (F14) and copper hydroxide 80% plus copper 50% (F26), plus azoxystrobin 25% (F3), were chosen for this study. The factors fungicide and temperature were significant in both grape infection and OTA production experiments, and the factor strain only in the OTA production trial (data not shown). The percentage of grapes infected by *A. carbonarius* was calculated for each treatment. Infection was significantly higher at 30°C.

### Table II. Mean growth rates (mm day⁻¹) and ochratoxin A (OTA) production on synthetic nutrient medium (SNM, μg g⁻¹) at two temperatures (20 and 30°C) by three strains of *Aspergillus carbonarius* (3.161, 3.162 and 3.168).

| Fungicide | 20°C | 30°C | 20°C | 30°C |
|-----------|------|------|------|------|
| Control   | 5.46±0.16±ab | 7.98±0.32±ab | 5.68±5.13±bc | 1.14±1.17±c |
| F1        | n.g. | n.g. | <d.l.d | <d.l.d |
| F2        | 1.73±0.15±ad | 1.67±0.52±ad | 19.66±16.24±a | 14.20±0.81±b |
| F3        | 4.50±0.33±bc | 7.79±1.77±b | 2.84±4.70±bc | 0.22±0.30±e |
| F4        | n.g. | n.g. | <d.l.d | <d.l.d |
| F5        | 1.53±0.63±cd | 3.00±0.43±f | 13.34±20.29±be | 2.58±3.01±c |
| F6        | n.g. | n.g. | <d.l.d | <d.l.d |
| F7        | 3.91±0.35±c | 6.77±0.10±c | 14.41±17.98±b | 0.64±0.59±e |
| F8        | 5.24±0.65±bc | 8.65±0.99±a | 8.03±7.00±bc | 8.92±9.55±bc |
| F9        | n.g. | n.g. | <d.l.d | <d.l.d |
| F10       | n.g. | n.g. | <d.l.d | <d.l.d |
| F11       | 2.26±1.56±d | 1.59±0.18±f | 11.29±12.74±bc | 58.61±18.23±a |
| F12       | n.g. | n.g. | <d.l.d | <d.l.d |
| F13       | 1.91±0.42±ed | 2.66±0.22±eg | 0.96±1.56±a | 0.34±0.50±d |
| F14       | 3.92±0.29±f | 6.34±0.43±g | 4.28±3.73±bc | 1.96±3.27±e |
| F15       | n.g. | n.g. | <d.l.d | <d.l.d |
| F16       | n.g. | n.g. | <d.l.d | <d.l.d |
| F17       | 5.53±0.05±bc | 7.58±0.83±bc | 1.24±2.14±c | 0.95±1.64±c |
| F18       | n.g. | n.g. | <d.l.d | <d.l.d |
| F19       | n.g. | n.g. | <d.l.d | <d.l.d |
| F20       | 2.20±0.70±d | 4.41±0.89±f | 1.05±1.72±c | 0.94±1.62±c |
| F21       | 0.85±0.46±f | 2.96±0.35±f | 0.07±0.12±e | 4.06±2.77±c |
| F22       | 5.72±0.81±a | 8.71±1.04±b | 6.82±6.94±bc | 3.96±5.43±bc |
| F23       | n.g. | n.g. | <d.l.d | <d.l.d |
| F24       | n.g. | n.g. | <d.l.d | <d.l.d |
| F25       | n.g. | n.g. | <d.l.d | <d.l.d |
| F26       | 2.64±0.30±d | 3.56±0.06±d | 1.97±2.52±bc | 1.86±3.22±c |

Values are the mean of the three strains and three replicates of each ± standard deviation (SD). Data in each column followed by different letters are significantly different for the Duncan test. n.g., No growth; <d.l., below the limit of detection.

*Minima doses preventing Aspergillus carbonarius growth on synthetic grape-like medium*

No significant differences were found between the two strains of *A. carbonarius* tested (data not shown). At the optimum temperature for *A. carbonarius* growth, 30°C, a higher concentration of fungicide was needed to prevent fungal growth than at 20°C. Each fungicide had a different effect on *A. carbonarius* growth, but most of them were effective at doses around one-quarter (d3) of the dose recommended by the manufacturer (D) (Table III). Only three fungicides did not prevent growth at this dose: F4 at D (d1) and F1 and F16 at 0.50 × D (d2), in the assays at 30°C.
Figure 1. Growth rate (mm day$^{-1}$) and ochratoxin A (OTA) production (µg g$^{-1}$) at (▲) 20°C and (■) 30°C of three strains of *Aspergillus carbonarius* (3.161, 3.162 and 3.168) after the addition of several fungicides (F1–F26) to the synthetic nutrient medium (SNM) medium. Values are the mean of the three strains. No fungicide was added to the control treatment and growth and OTA production was considered as zero.

Table III. *Aspergillus carbonarius* growth at 20 and 30°C on synthetic nutrient medium (SNM) containing different fungicides at different doses: $D_i$ dose recommended by the manufacturer; $d_1$, 0.75 × $D_i$; $d_2$, 0.5 × $D_i$; $d_3$, 0.25 × $D_i$; $d_4$, 0.1 × $D_i$; $d_5$, 0.01 × $D_i$; and $d_6$, 0.005 × $D_i$.

| Fungicide | $20^\circ C$ | $30^\circ C$ | $20^\circ C$ | $30^\circ C$ | $20^\circ C$ | $30^\circ C$ | $20^\circ C$ | $30^\circ C$ | $20^\circ C$ | $30^\circ C$ | $20^\circ C$ | $30^\circ C$ |
|-----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Control   | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           |
| F1        | –           | –           | –           | –           | +           | +           | +           | +           | +           | +           | +           | +           |
| F4        | –           | –           | –           | –           | +           | +           | +           | +           | +           | +           | +           | +           |
| F6        | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           | +           |
| F9        | –           | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           |
| F10       | –           | –           | –           | –           | –           | –           | –           | –           | –           | –           | –           | –           |
| F12       | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           |
| F15       | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           |
| F16       | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           |
| F18       | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           |
| F19       | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           |
| F23       | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           |
| F24       | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           |
| F25       | –           | –           | –           | –           | –           | –           | –           | +           | +           | +           | +           | +           |

+, *A. carbonarius* growth; –, no growth.
than at 20°C. A percentage of reduction of the percentage of infection for each treatment was determined by comparison with the control. The average of the percentage of reduction of both strains is shown in Figure 2. Infection was reduced at both temperatures with azoxystrobin 25% (F3) and cyprodinil 37.5% plus fludioxonil 25% (F9), and with penconazole 10% (F1) at 20°C. Maximum reduction (>95%) was achieved with cyprodinil 37.5% plus fludioxonil 25% (F9) at 20°C, followed by azoxystrobin 25% (F3) (65%) and penconazole 10% (F1) (59%) at the same temperature. Around 10% reduction in the infection percentage was also detected with mancozeb 80% (F23) at 20°C. In general, fenhexamid 50% (F14), mancozeb 80% (F23) and copper hydroxide 80% plus copper 50% (F26) enhanced A. carbonarius grape infection, especially at 30°C.

Half of the fungicides (penconazole 10%, azoxystrobin 25% and cyprodinil 37.5% plus...
fludioxonil 25%; F1, F3 and F9) showed a clear reduction of the OTA production at both temperatures in comparison with the control treatment (Figure 3). For these fungicides, reduction was higher at 20°C than at 30°C, except for penconazole 10% (F1) for 3.162 strain. Differences in the percentage of reduction were also observed between both strains, especially at 30°C, where fungicides were more effective against strain 3.168. At 20°C, fenhexamid 50% (F14), mancozeb 80% (F23) and copper hydroxide 80% plus copper 50% (F26) reduced the OTA production for strain 3.168 meanwhile they produced the opposite effect on strain 3.162, increasing OTA production more than 200% sometimes. At 30°C, these three fungicides increased up to 20% OTA production of both strains.

**Discussion**

A significant effort has been concentrated on the development and use of fungicides for the control of other food-spoilage fungi such as *Fusarium* spp. (Moss and Frank 1985; Mathies and Buchenauer 1996), *Aspergillus flavus* and *A. ochraceus* (Munimbazi et al. 1997), *Botrytis cinerea* (Slawecki et al. 2002), etc., but not on studies on grapes. Existing earlier studies have shown that combinations of Euparen (a sulphamide-type fungicide) and Mycodifol (Karadimcheva 1978), or captan (Tandon et al. 1975) were found to be effective against black aspergilli colonizing grape berries. Data on the resistance of *A. carbonarius* to fungicide treatments is non-existent so far. Therefore, the efficiency against this mould of a range of fungicides designed to control other species infecting vines was tested in the present study. Moreover, it has to be underlined that the doses proposed by the manufacturers and used in the present study were also designed for the control of other moulds.

To discuss the results obtained, the different fungicides may be grouped according to their active ingredients. *A. carbonarius* growth and OTA production were minimized when using fungicides with copper in their composition (F20, F21, F24, F25 and F26). However, copper oxychloride was also present in F5, which although limiting *A. carbonarius* growth, did not decrease OTA production. The same OTA-enhancing effect was detected for fluazinam (F2) and procymidone (F11), which were classified as dinitro aniline and dicarboximide fungicides, respectively. It is known that fluazinam (F2), together with azoxystrobin (F3, F13 and F17), thifluzamide and carboxin can interfere with respiration processes (Corbett et al. 1984; Guo et al. 1991; Sauter et al. 1995). Both *A. carbonarius* growth and OTA production were increased, although not significantly, when adding inorganic fungicides containing sulfur to the medium (F8 and F22). Similar effects were detected in a study of the OTA content in red wines produced from vineyards treated with different pesticides (Lo Curto et al. 2004). The level of OTA in wines from sulphur-treated grapes was higher than in the other samples. Furthermore, those authors reported azoxystrobin as a fungicide able to reduce OTA concentration in wine, with 96.5% of reduction. Data on OTA concentration in wine are not directly comparable with the screening in this study because OTA concentration in wine or grape accounts for total OTA. In the present study, OTA-producing capacity must be coupled to colony size data to let one have an idea of the total OTA accumulation. Thus, in the present study, the total amount of OTA produced by *A. carbonarius* growing with sulphur fungicides increased due to both the higher OTA-producing capacity detected and the bigger diameters of the colonies. However, it is not clear whether the total amount of OTA accumulated by *A. carbonarius* treated with fluazinam (F2) and procymidone (F11) increased because OTA production stimulation occurred but, in contrast, smaller colonies were observed. Some other fungicides have been found to stimulate OTA production in grapes (Battilani et al. 2003).

Little is known about the mechanism of action of the active ingredients of the fungicides assayed. For many compounds, spore germination is the growth stage that is most sensitive to inhibition (Slawecki et al. 2002). In the present study, fungicides that completely inhibited germination were enclosed in several groups according to their active ingredients: amide (F12, one component, and F15) and dicarboximide fungicides (F12, one component, and F18), triazol fungicides (F1 and F16), benzimidazole (F19) and dithiocarbamate fungicides (F23, F24 and F25, one component), pyrimidine fungicides (F9, one component, and F10), phenylpyrrole fungicides (F6 and F9, one component), etc. It would be interesting to study growth for longer periods in order to know if this last group of fungicides at the doses assayed, totally inhibited growth or only prolonged the lag phase of the mould. Doses of these fungicides were reduced in a subsequent experiment in order to find the threshold dose preventing *A. carbonarius* growth. In general, fungicides with the same active ingredients seemed to have similar effects when reducing the doses. Triazol fungicides (F1 and F16) were the less effective fungicides against *A. carbonarius* growth, as just when reducing up to one-quarter of the initial dose the inhibitory growth effect disappeared. Fungicides with the MIC at one-quarter of the initial one (F19, F23, F24 and F25) were classified as carbamate and
dithiocarbamate fungicides. Cyprodinil seemed to be the active ingredient more suitable to stop fungal growth as it was a component of the pyrimidine fungicides F9 (Switch) and F10 (Chorus), which showed the minimum threshold concentrations. Conversely, Greek authors observed that pesticides such as Carbendazim and Chorus were ineffective in controlling sour rot caused by aspergilli (Tjamos et al. 2004). However, the application of Switch led to a significant decrease in the incidence of black aspergilli on grapes. The fungicide Switch contains cyprodinil and fludioxonil, which belong to the pyrimidine and pyrrolnitrin classes of fungicides, respectively. Since the fungicide Chorus contains cyprodinil and was ineffective against aspergilli, it was concluded that fludioxonil was the active ingredient of Switch (Tjamos et al. 2004).

Sauter et al. (1995) noted that the group of fungicides containing the strobilurins, blocked electron transport at the cytochrome bc1 complex of the mitochondrial electron transport chain, and therefore were extremely potent inhibitors of spore germination, but much less active as inhibitors of mycelial growth. No germination-inhibitory effect of fungicides grouped as strobilurin fungicides (F3, F13 and F17) was noticeable in this study, as the three of them allowed A. carbonarius growth, although less than the control treatment. Other reported fungicides that typically acted after germination in filamentous fungi by strongly inhibiting mycelial growth, included antimicotubule agents (carbendazim and N-phenylcarbamates, which inhibited nuclear division; Suzuki et al. 1984), and inhibitors of ergosterol biosynthesis (Buchenauer 1987). However, in the present study, benzimidazol (F19) and dithiocarbamate fungicides (F23, F24 and F25, one component) showed a completely inhibition of germination as mentioned above.

On grapes, a mixture of cyprodinil (37.5%) and fludioxonil (25%) (F9) seemed the best fungicide to control A. carbonarius growth and OTA production together with penconazole 10% (F1) and azoxyostrobin 25% (F3). All three were also restrictive fungicides in terms of growth and mycotoxin production when tested on SNM medium. Penconazole was previously reported as a synthetic pesticide able to reduce around 90% the level of OTA in wines made from grapes treated with this fungicide (Lo Curto et al. 2004). In another study carried out at the ITV France by Molot and Solanet (2003), the fungicides Switch (F9), Scala (containing the pyrimidine fungicide pyrimethanil) and Mikal (containing fosetyl-Al and the dicarboximide folpel) were found to be the most effective for lowering fungal colonization and OTA content of wines. Fenhexamid 50% (F14) and copper 50% (F26) showed the same effects on synthetic nutrient medium than on grapes, as they increased OTA production, especially at 30°C. Temperature was a determinant factor and could influence the effectiveness of the fungicides. Results are in accordance with previous work, reporting optimum temperatures for A. carbonarius growth and OTA production at 30 and 20°C, respectively (Belli et al. 2005).

Propitious levels of other environmental factors, such as humidity, could also interfere in the efficacy of the fungicides assayed, together with the reiterative application of the same fungicide, as it could modify the equilibrium in the ecosystem, enhancing other microorganisms development as competing fungi are removed.

Additional in vitro studies on grapes testing the whole range of fungicides are needed in order to find out the best active ingredients against A. carbonarius development and mycotoxin production. Afterwards, further studies of the in situ efficiency of pesticide treatments against A. carbonarius infection and OTA production in vines would be required.

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