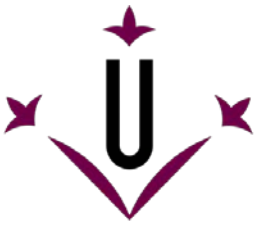

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Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes

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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 (OTA) production. Three trials were designed in order to: i) screen 26 fungicides at the doses recommended by manufacturers on grape-like synthetic medium (SNM) at 20 ºC and 30 ºC; ii) find out the minimum inhibitory concentration (MIC) of each fungicide for *A. carbonarius* growth on synthetic medium, and iii) investigate the effect of several fungicides on *A. carbonarius*-inoculated grapes. In synthetic medium, nine fungicides significantly reduced *A. carbonarius* growth rate, meanwhile 13 completely inhibited its growth. In general, growth was faster at 30 ºC than at 20 ºC, contrary to OTA production. Fungicides that stopped fungal growth also inhibited OTA production, but not all the fungicides that reduced growth reduced the OTA synthesis. In general, fungicides that contained copper or strobilurins reduced both growth and OTA production, contrary to sulfur fungicides. At the optimum temperature for *A. carbonarius* growth of 30 ºC, higher amounts of fungicide were needed to prevent fungal growth than at 20 ºC. Among the fungicides that inhibited *A. carbonarius* growth on SNM at the initial doses, cyprodinil seemed to be the active ingredient more effective to stop fungal growth when testing reduced doses. Fungicide effect on grapes was similar to that on synthetic medium. Both infection and OTA production were reduced when using cyprodinil (37.5%) + fludioxonil (25%) and azoxystrobin (25%). Penconazole (10%) also showed a clear reduction in OTA production at both temperatures, although infection was only reduced at 20 ºC. OTA reduction was strain and temperature-dependent. In general, fenhexamid (50%), mancozeb (80%) and copper hydroxide (80%) + copper (50%) enhanced infection and OTA production.

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Keywords: fungicides, *A. 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 is one of the more studied mycotoxins in wines nowadays, being recently regulated in the European Union (Commission Regulation 123/2005), mainly due to its high toxicity and its presence in wines over the world. Its production in grapes from Mediterranean area is associated with different *Aspergillus* spp., mostly black aspergilli and among them, *A. carbonarius* (Cabañes *et al*. 2001, Battilani *et al*. 2003, Bellí *et al*. 2004a).

Prevention of 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 the mould growth (Munimbazi *et al*. 1997).

The aim of this 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 to: i) screen the main fungicides used in Spanish vines, to test their efficiency against *A. carbonarius* growth and OTA production on synthetic nutrient medium (SNM); ii) find out the minimum inhibitory concentration (MIC) of

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each fungicide for *A. carbonarius* growth on SNM; iii) investigate the effect of several fungicides on growth and OTA production of *A. carbonarius* inoculated on grapes.

Material 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 centrally inoculate (10^6 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 a_w (Bellí et al. 2004b). Strains are 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 1). Each fungicide was aseptically added to the autoclaved medium before plating it. No fungicides were added to control plates. Plates were incubated at 20 ºC and 30 ºC 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, Massachusetts, U.S.A.) (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 C_{18} column (Waters Spherisorb 5 µm, ODS2, 4.6x250 mm) was used. The injection volume and the 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

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ratio of 3:1. The ochratoxin A standard was from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). The standard solution was made in methanol and concentration confirmed by using an UV spectrophotometer. Three repetitions were carried out, both for growth and OTA studies.

[Insert table 1 about here]

Minimum doses preventing A. 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 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 10^6 spores ml^{-1} for use as inoculum. SNM plates (0.99 a_w) with decreasing concentrations of those fungicides (D, dose recommended by the manufacturer; d1, 0.75xD; d2, 0.5xD; d3, 0.25xD; d4, 0.1xD; d5, 0.01xD and d6, 0.005xD) were single-point inoculated and incubated at 20 ºC and 30 ºC. Growth was measured daily during a period of 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 (0.1xD). Table grapes (Red Globe var.) were surface disinfected by dipping them in NaClO (0.1 % Cl) and ethanol (70 %) solutions for 30 seconds and excess of moisture was aseptically removed. Afterwards, 20 grapes were dipped in a fungicide solution for 30 seconds and were placed onto a grid inside plastic boxes containing 300

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ml of water to keep a high relative humidity (90-100%) throughout the experiment. In control treatments, grapes were dipped in water instead of fungicide. For culturing, 10^3 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 ºC and 30 ºC, the percentage of grapes infected by *A. carbonarius* was assessed. The whole set of grapes of each treatment were crushed, and filtered through Whatman nº1 filter paper under vacuum. OTA was extracted from this must following the method of Bezzo *et al*. (2000). Twenty-five µl of each sample were injected into the HPLC system equipped with a fluorescence detector (Waters 474, Milford, Massachusetts, U.S.A.) (λ_{exc} 230 nm; λ_{em} 458 nm) and a C_{18} column (Waters Spherisorb 5 µm, ODS2, 4.6x250 mm). The analysis was performed under isocratic conditions, with acetonitrile 48% -sodium acetate 4mM/acetic acid (19/1)- 52%, as the mobile phase, pumped at a flow rate of 1 ml min^{-1}. The injection volume and the retention time were 25 µl and 12 min, respectively. The detection limit of the analysis was 0.05 µg l^{-1}, based on a signal: 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 UV spectrophotometer.

Statistical treatment of the results

The regression lines of colony diameters against days after inoculation were calculated for each fungicide and were 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 statistically analysed with SAS Enterprise Guide software (SAS Institute, version 2.0, Inc., Cary, N.C., U.S.A.) by analysis of variance

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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 ºC than at 20 ºC, 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 thirteen fungicides completely inhibited fungal growth at the dose assayed (F1, F4, F6, F9, F10, F12, F15, F16, F18, F19, F23, F24 and F25) (Table 2). 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, either in their response to temperature and fungicide treatments. The interaction fungicide x temperature was also significant. Contrary to the growth pattern, OTA production was in general higher at 20 ºC than at 30 ºC. 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

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F11, which favoured toxin production at both temperatures. OTA was also favoured by the addition of F7 at 20 ºC and F14, F21 and F26 at 30 ºC. OTA was also higher than the control under the effect of F8 at both temperatures although growth was only stimulated at 30 ºC. Contrary, F17 reduced OTA production although growth was favoured at 20 ºC. OTA was favoured at 20 ºC and 30 ºC under the effect of F22, the unique fungicide that increased fungal growth at both temperatures. Figure 1 compares the growth rate and the 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 a reduction of both growth and OTA production.

[Insert table 3 and figure 1 about here]

Minima doses preventing A. 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 ºC, higher concentration of fungicide was needed to prevent fungal growth than at 20 ºC. Each fungicide had a different effect on *A. carbonarius* growth, but most of them were effective at doses around $1/4^{th}$ (d3) of the dose recommended by the manufacturer (D) (Table 3). Only three fungicides did not prevent growth at this dose: F4 at D (d1) and F1 and F16 at 0.50 x D (d2), in the assays at 30 ºC. $1/10^{th}$ of D (d4), was the MIC of fungicides F4 and captan 50% (F18) at 20 ºC, and fludioxonil (F6) and tolyfluanid (F15) at both temperatures. A mixture of cyprodinil 37.5% and fludioxonil 25% (F9)

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and cyprodinil alone (F10), were the most effective fungicides, as they hinder growth at the minimum doses assayed (d5 and d6, respectively).

[Insert table 3 about here]

Effect of fungicides on grapes

Three fungicides that completely inhibited *A. carbonarius* on SNM: penconazole 10% (F1), cyprodinil 37.5% + 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%+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 ºC than at 20 ºC. 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%+fludioxonil 25% (F9), and with penconazole 10% (F1) at 20 ºC. Maximum reduction (> 95 %) was achieved with cyprodinil 37.5%+fludioxonil 25% (F9) at 20 ºC, 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 ºC. In general, fenhexamid 50% (F14), mancozeb 80% (F23) and copper hydroxide 80%+copper 50% (F26) enhanced *A. carbonarius* grape infection, especially at 30 ºC.

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[Insert figure 2 about here]

Half of the fungicides (penconazole 10%, azoxystrobin 25% and cyprodinil 37.5%+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 ºC than at 30 ºC, except for penconazole 10% (F1) for 3.162 strain. Differences in the percentage of reduction were also observed between both strains, especially at 30 ºC, where fungicides were more effective against strain 3.168. At 20 ºC, fenhexamid 50% (F14), mancozeb 80% (F23) and copper hydroxide 80%+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 ºC, these three fungicides increased up to 20 % OTA production of both strains.

[Insert figure 3 about here]

Discussion

A significant effort has been concentrated on the development and use of fungicides for the control of other food-spoilage fungi like *Fusarium* spp. (Mathies and Buchenauer 1996, Moss and Frank 1985), *Aspergillus flavus* and *A. ochraceus* (Munimbazi *et al.* 1997), *Botrytis cinerea* (Slawecki et al. 2002), etc, but not any studies on grapes. Existing earlier studies have shown that combinations of Euparen (a sulfamide type fungicide) and Mycodifol (Karadimcheva 1978), or captan (Tandon et al. 1975) were found to be effective against black aspergilli colonising grape berries. Data on resistance of *A. carbonarius* to fungicide treatments is non-existent so far. Therefore,

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the efficiency against this mould of a range of fungicides designed to control other species infecting vines, was tested in this 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 minimised 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 sulfur-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 to the screening in this study, because OTA concentration in wine or grape accounts for total OTA. In our study, both OTA producing capacity must be coupled to colony size data to have an idea of the total OTA accumulation. Thus, in the present study, the total amount of OTA produced by *A. carbonarius* growing with

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sulfur 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 or not because OTA production stimulation occurred but, in contrast, smaller colonies were observed. Some othe 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-, F15) and dicarboximide fungicides (F12 –one component-, 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 reducing up to one quarter 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

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to stop fungal growth, as it was a component of the pyrimidine fungicides F9 (Switch) and F10 (Chorus), which showed the minimum threshold concentrations. Oppositely, 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 significant decrease in 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 bc_1 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 antimicrotubule agents (carbendazim and N-phenylcarbamates, which inhibited nuclear division (Suzuki *et al*. 1984)), and inhibitors of ergosterol biosynthesis (Buchenauer, 1987). However, in this study, benzimidazol (F19) and dithiocarbamate fungicides (F23, F24 and F25 –one component-) showed a completely inhibition of germination as mentioned before.

On grapes, a mixture of cyprodinil (37.5 %) and fludioxonil (25 %) (F9) seemed the best fungicide that controlled *A. carbonarius* growth and OTA production together with

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penconazole 10% (F1) and azoxystrobin 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 ºC. Temperature was a determinant factor and could influence in the effectiveness of the fungicides. Results are in accordance with previous work, reporting optimum temperatures for *A. carbonarius* growth and OTA production at 30 ºC and 20 ºC, respectively (Bellí *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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References

BATTILANI, P., PIETRI, A., BERTUZZI, T., LANGUASCO, L., GIORNI, P., and KOZAKIEWICZ, Z., 2003, Occurrence of Ochratoxin A-producing fungi in grapes grown in Italy. *Journal of Food Protection*, **66**, 633-636.

BELLÍ, N., PARDO, E., MARÍN, S., FARRÉ, G., RAMOS, A.J., and SANCHIS, V., 2004a, Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *Journal of the Science of Food and Agriculture*, **84**, 541-546.

BELLÍ, N., MARÍN, S., SANCHIS, V., and RAMOS, A.J., 2004b, Influence of water activity and temperature on growth of isolates of *Aspergillus* section *Nigri* obtained from grapes. *International Journal of Food Microbiology*, **96**, 19-27.

BELLÍ, N., RAMOS, A.J., CORONAS, I., SANCHIS, V., and MARÍN, S., 2005, *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape

For Peer Review Only

medium in relation to environmental factors. *Journal of Applied Microbiology*, **98**, 839-844.

BEZZO, G., MAGGIOROTTO, G., and TESTA, F., 2000, *A method for the determination of specific mycotic contaminants random occurring in wines. FV Nº 1097, 2706/070200*, (Paris: Office International de la Vigne et du Vin).

BRAGULAT, M.R., ABARCA, M.L., and CABAÑES, F.J., 2001, An easy screening method for fungi producing ochratoxin A in pure culture. *International Journal of Food Microbiology*, **71**, 139-144.

BUCHENAUER, H., 1987, Mechanism of action of triazolyl fungicides and related compounds. *Modern selective fungicides: properties, applications, mechanisms of action*, edited by H. Lyr, (Harlow, U.K.: Longman Scientific and Technical), pp. 205-231.

CABAÑES, F.J., ACCENSI, F., BRAGULAT, M.R., ABARCA, M.L., CASTELLÁ, G., MINGUEZ, S., and PONS, A., 2001, What is the source of ochratoxin A in wine?. *International Journal of Food Microbiology*, **79**, 213-215.

COMMISSION REGULATION (EC), 2005, *No* 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2001 regarding ochratoxin A, *Official Journal of European Communities*, **L25**, 3-5.

CORBETT, J.R., WRIGHT, K., and BAILLIE, A.C., 1984, The biochemical mode of action of pesticides, p. 291-309. Academic Press, London, United Kingdom.

For Peer Review Only

GUO, Z.J., MIYOSHI, H., KOMYOJI, T., HAGA, T., and FUJITA, T., 1991, Uncoupling activity of a newly developed fungicide, fluazinam [3-chloro-N-(3-chloro-2,6-dinitro-4-trifluoromethylphenyl)-5-trifluoromethyl-2-pyridinamine]. *Biochimica et Biophysica Acta*, **1056**, 89-92.

KARADIMCHEVA, B., 1978, Effect of some fungicides on the epiphytic microflora in grapes. *Gradinar Lozar Nauka*, **15**, 159-166.

LO CURTO, R., PELLICANÒ, T., VILASI, F., MUNAFÒ, P., and DUGO, G., 2004, Ochratoxin A occurrence in experimental wines in relationship with different pesticide treatments on grapes. *Food Chemistry*, **84**, 71-75.

MATHIES, A., and BUCHENAUER, H., 1996, Investigations on the action of different active ingredients on the biosynthesis of mycotoxins in *Fusarium culmorum* and *F. graminearum*. *Modern fungicides and antifungal compounds*, edited by H. Lyr, P.E. Russell, and H.D. Sisler (Andover: Intercept Ltd.), pp. 199-204.

MOLOT, B., and SOLANET, D, 2003, Ochratoxine: prévention du risque. Etude au vignoble de fongicides actifs contre Aspergillus carbonarius incidences sur la présence aux vendanges. *Les Entretiens Viti-Vinicoles Rhone-Mediterranée*, Nimes, 16 avril 2003, pp. 18-21.

MOSS, M.O., and FRANK, M., 1985, The influence of the fungicide tridemorph on T-2 toxin production by *Fusarium sporotrichioides*. *Transactions of the British Mycological Society*, **84**, 585-590.

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412

413 MUNIMBAZI, C., SAXENA, J., TSAI, W.J., and BULLERMAN, L.B., 1997,
414 Inhibition of production of cyclopiazonic acid and ochratoxin A by the fungicide
415 iprodione. *Journal of Food Protection*, **60**, 849-852.

416

417 SAUTER, H., AMMERMANN, E., BENOIT, R., BRAND, S., GOLD, R.E.,
418 GRAMMENOS, W., KOEHLE, H., LORENZ, G., MUELLER, B., ROEHL, F.,
419 SCHIRMER, U., SPEAKMAN, J.B., SHERALD, J.L., RAGSDALE, N. N., and
420 SISLER, H.D., 1995, Similarities between the systemic fungicides triforine and
421 triarimol. *Pesticide Science*, **4**, 719-728.

422

423 SLAWECKI, R.A., RYAN, E.P. and YOUNG, D.H., 2002, Novel fungitoxicity assays
424 for inhibition of germination –associated adhesion of *Botrytis cinerea* and *Puccinia*
425 *recondite* spores. *Applied and Environmental Microbiology*, **68**, 597-601.

426

427 SUZUKI, K., KATO, T., TAKAHASHI, J., and KAMOSHITA, K., 1984, Mode of
428 action of methyl *N*-(3,5-dichlorophenyl)-carbamate in the benzimidazole-resistant
429 isolate of *Botrytis cinerea*. *Journal of Pesticide Science*, **9**, 497-501.

430

431 TANDON, M.P., JAMALUDDIN, and BHARGAVA, V., 1975, Chemical control of
432 decay of fruit of *Vitis vinifera* caused by *Aspergillus niger* and *Penicillium* sp. *Current*
433 *Science*, **44**, 478.

434

435 TJAMOS, S.E., ANTONIOU, P.P., KAZANTZIDOU, A., ANTONOPOULOS, D.F.,
436 PAPAGEORGIOU, I., and TJAMOS, E.C., 2004, *Aspergillus niger* and *A. carbonarius*

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437 in Corinth raisin and wine-producing vineyards in Greece: population composition,
438 ochratoxin A production and chemical control. *Journal of Phytopathology*, **152**, 250-
439 255.

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Table 1. Fungicides used in the study, their composition, origin and doses recommended by the manufacturers.

Code	Fungicide	Company	Composition	Dose
F1	TOPAS	Syngenta	Penconazole 10% p/v	0.35 ml l^{-1}
F2	SHIRLAN 500SC	Syngenta	Fluazinam 50% p/v	2 ml l^{-1}
F3	QUADRIS	Syngenta	Azoxystrobin 25 % p/v	2.25 ml l^{-1}
F4	Experimental product	Syngenta	CGA302130	2 ml l^{-1}
F5	CUPROCOL	Syngenta	Copper oxychloride 70% p/v	2 ml l^{-1}
F6	GEOXE	Syngenta	Fludioxonil 50%	0.5 g l^{-1}
F7	Experimental product	Syngenta	CGA379438	1 g l^{-1}
F8	THIOVIT JET	Syngenta	Sulfur 80% WG	4 g l^{-1}
F9	SWITCH	Syngenta	Cyprodinil 37.5% + Fludioxonil 25%	1.8 g l^{-1}
F10	CHORUS 50 WG	Syngenta	Cyprodinil 50%	2 g l^{-1}
F11	SUMISCLEX 50WP	Masso	Procymidone	1 g l^{-1}
F12	RIDOMIL GOLD COMBI	Syngenta	Folpet 40% + Mefenoxam 5% WP	2 g l^{-1}
F13	QUADRIS DUO	Syngenta	Azoxystrobin 18.7% + Cymoxanil 12% WG	2.25 g l^{-1}
F14	TELDOR	Bayer	Fenhexamid 50% p/p	2 g l^{-1}
F15	EUPAREN M	Bayer	Tolyfluanid 50% p/p	1.75 g l^{-1}
F16	FOLICUR 25EW	Bayer	Tebuconazole 25% p/v	0.70 ml l^{-1}
F17	FLINT	Bayer	Trifloxystrobin 50% p/p	0.13 g l^{-1}
F18	CAPLUQ-50	Luqsa	Captan 50% p/p	3.5 g l^{-1}

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F19	CARBENLUQ-50	Luqsa	Carbendazim 50% p/p	0.6 g l^{-1}
F20	COBRELUQ-50	Luqsa	Copper oxychloride 50% p/p	3.5 g l^{-1}
F21	CUPROLUQ	Luqsa	Cuprous oxide 75% p/p	2 g l^{-1}
F22	LUQSAZUFRE	Luqsa	Sulfur 80% p/p	5 g l^{-1}
F23	MANCOZEB 80	Luqsa	Mancozeb 80% p/p	3 g l^{-1}
F24	TMTD 80	Luqsa	Tiram 80% p/p	2.5 g l^{-1}
F25	ZICOLUQ 320	Luqsa	Copper oxychloride 22% p/p + Mancozeb 17.5% p/p	5 g l^{-1}
F26	HIDROXILUQ 800	Luqsa	Copper hydroxide 80% p/p + Copper 50 % p/p	2 g l^{-1}

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Table 2. Mean growth rates (mm day^{-1}) and OTA production on Synthetic Nutrient Medium (μg g^{-1} SNM) at two temperatures (20 ºC and 30 ºC) by three strains of *A. carbonarius* (3.161, 3.162 and 3.168). Values are the mean of the three strains and three replicates of each ± standard deviation. Data in each column followed by different letters are significantly different in the Duncan test.

	Growth rate (mm day^{-1})		OTA production (μg g^{-1} SNM)	
Fungicide	20 ºC	30 ºC	20 ºC	30 ºC
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^{ed}	1.67 ± 0.52^{fg}	19.66 ± 16.24^{a}	14.20 ± 0.81^{b}
F3	4.50 ± 0.32^{bc}	7.79 ± 1.77^{ab}	2.84 ± 4.70^{bc}	0.22 ± 0.39^{c}
F4	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F5	1.53 ± 0.63^{ed}	3.00 ± 0.43^{ef}	13.34 ± 20.29^{abc}	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^{bc}	14.41 ± 17.98^{ab}	0.64 ± 0.59^{c}
F8	5.24 ± 0.65^{ab}	8.65 ± 0.99^{a}	8.03 ± 7.00^{abc}	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^{g}	11.29 ± 12.74^{abc}	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^{efg}	0.96 ± 1.56^{c}	0.34 ± 0.59^{c}
F14	3.92 ± 0.29^{c}	6.34 ± 0.43^{c}	4.28 ± 3.73^{bc}	1.96 ± 3.27^{c}
F15	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}
F16	N.G.	N.G.	<d.l.^{d}	<d.l.^{d}

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F17	5.53 ± 0.05^{ab}	7.58 ± 0.83^{abc}	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^{d}	1.05 ± 1.72^{c}	0.94 ± 1.62^{c}
F21	0.85 ± 0.46^{e}	2.96 ± 0.35^{ef}	0.07 ± 0.12^{c}	4.06 ± 2.77^{c}
F22	5.72 ± 0.81^{a}	8.71 ± 1.04^{a}	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^{ed}	1.97 ± 2.52^{bc}	1.86 ± 3.22^{c}

450 N.G. no growth; <d.l. below detection limit.

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Table 3. *A. carbonarius* growth at 20 ºC and 30 ºC on SNM containing different fungicides at different doses: D, dose recommended by the manufacturer; d1, 0.75xD; d2, 0.5xD; d3, 0.25xD; d4, 0.1xD; d5, 0.01xD; d6, 0.005xD.

	D		d1		d2		d3		d4		d5		d6	
Fungicide	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC
Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F1	-	-	-	-	-	-	-	+	+	+	+	+	+	+
F4	-	-	-	+	-	+	-	+	-	+	+	+	+	+
F6	-	-	-	-	-	-	-	-	-	-	+	+	+	+
F9	-	-	-	-	-	-	-	-	-	-	-	+	+	+
F10	-	-	-	-	-	-	-	-	-	-	-	-	-	+
F12	-	-	-	-	-	-	-	-	+	+	+	+	+	+
F15	-	-	-	-	-	-	-	-	-	-	+	+	+	+
F16	-	-	-	-	-	-	-	+	+	+	+	+	+	+
F18	-	-	-	-	-	-	-	-	-	+	+	+	+	+
F19	-	-	-	-	-	-	-	-	+	+	+	+	+	+
F23	-	-	-	-	-	-	-	-	+	+	+	+	+	+
F24	-	-	-	-	-	-	-	-	+	+	+	+	+	+
F25	-	-	-	-	-	-	-	-	+	+	+	+	+	+

+ *A. carbonarius* growth; – no growth.

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Figure 1. Growth rate (mm day^{-1}) and OTA production (µg g^{-1}) at ▲20 ºC and ■30 ºC of three strains of *A. carbonarius* (3.161, 3.162 and 3.168) after the addition of several fungicides (F1-F26) to the 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.

Figure 2. Percentage of reduction in the percentage of grape infection by *A. carbonarius* treated with different fungicides and incubated at □20 ºC and ■30 ºC. Values are the mean of two strains.

Figure 3. Percentage of reduction of OTA production by two *A. carbonarius* strains (3.162 and 3.168) inoculated on grapes (µg ml^{-1} must) treated with different fungicides and incubated at □20 ºC and ■30 ºC.

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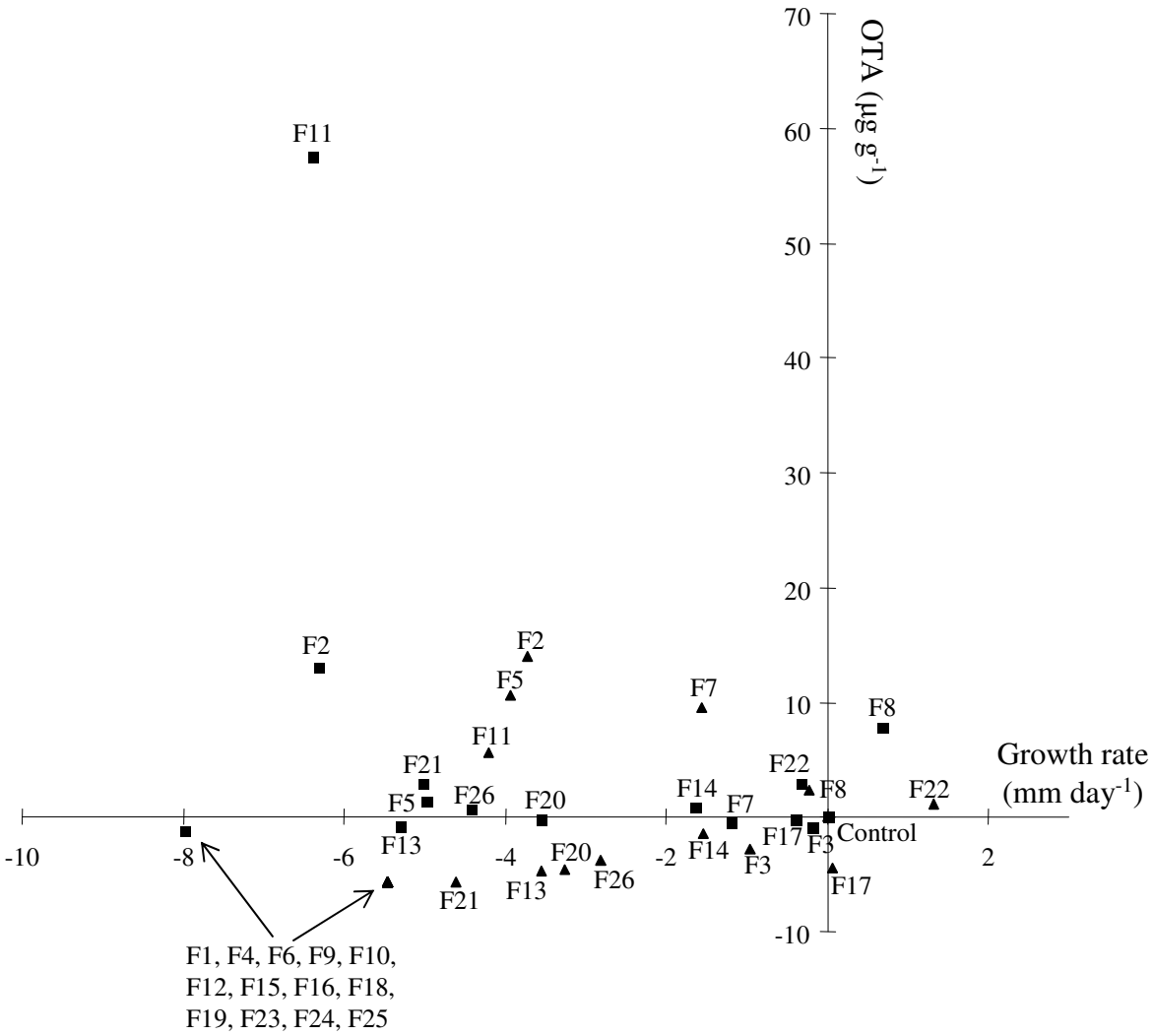
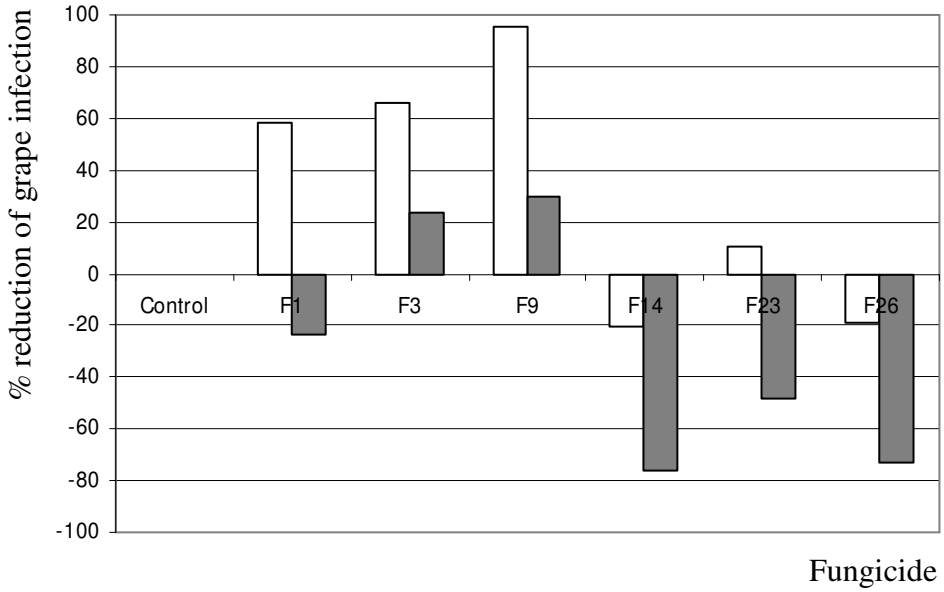
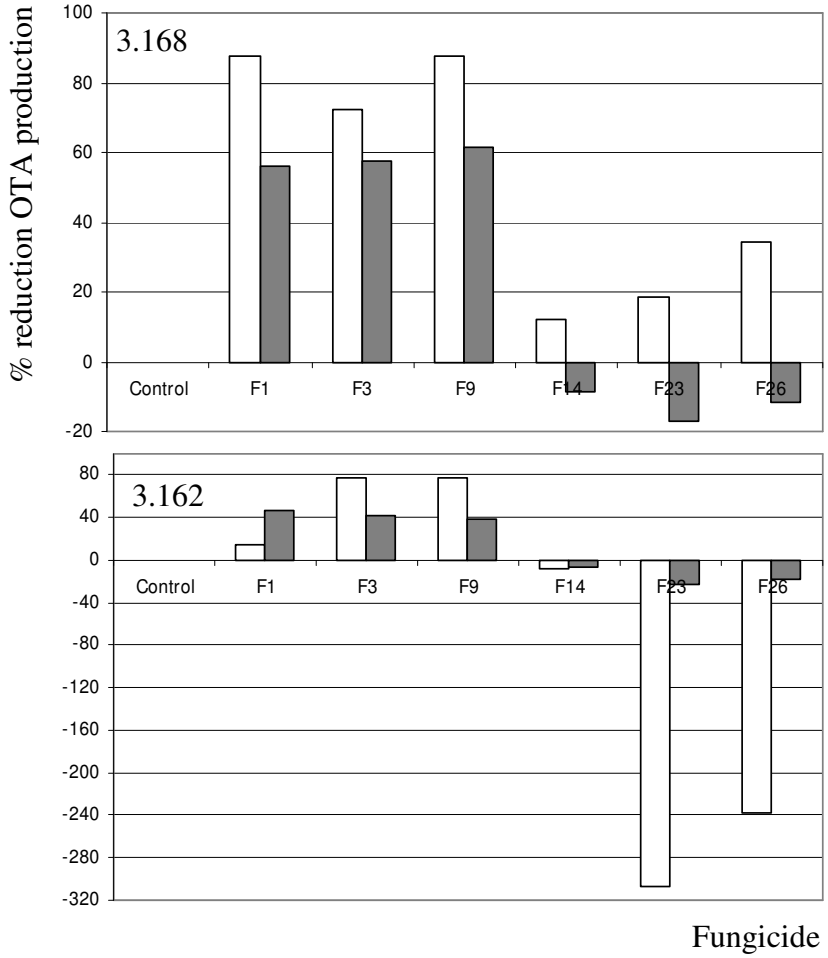

OTA (µg g-1)
70
60
50
40
30
20
10
-10
Growth rate
(mm day-1)
-10
-8
-6
-4
-2
2
F11
F2
F2
F5
F7
F8
F11
F21
F22
F5
F26
F20
F14
F7
F8
F22
F17
F3
Control
F13
F20
F14
F3
F26
F13
F21
F17
F1, F4, F6, F9, F10,
F12, F15, F16, F18,
F19, F23, F24, F25


% reduction of grape infection
100
80
60
40
20
0
-20
-40
-60
-80
-100
Control
F1
F3
F9
F14
F23
F26
Fungicide


% reduction OTA production
3.168
100
80
60
40
20
0
-20
Control
F1
F3
F9
F14
F23
F26
3.162
80
40
0
-40
-80
-120
-160
-200
-240
-280
-320
Control
F1
F3
F9
F14
F23
F26
Fungicide