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

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3 **1 Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin**

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6 **2 A production on synthetic grape-like medium and on grapes**

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15 **Abstract**

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

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3 40 **Keywords:** fungicides, *A. carbonarius*, ochratoxin A, grapes.
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8 42 **Introduction**
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10 43
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12 44 Ochratoxins are fungal secondary metabolites produced mainly by fungi from the
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14
15 45 genera *Aspergillus* and *Penicillium*, which are present in a wide variety of foods.
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17 46 Ochratoxin A is one of the more studied mycotoxins in wines nowadays, being recently
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20 47 regulated in the European Union (Commission Regulation 123/2005), mainly due to its
21
22 48 high toxicity and its presence in wines over the world. Its production in grapes from
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24
25 49 Mediterranean area is associated with different *Aspergillus* spp., mostly black aspergilli
26
27 50 and among them, *A. carbonarius* (Cabañes *et al.* 2001, Battilani *et al.* 2003, Bellí *et al.*
28
29 51 2004a).
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34 53 Prevention of growth of mycotoxin-producing fungi is the most effective strategy for
35
36 54 controlling the presence of mycotoxins in foods. This could be achieved by knowing the
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39 55 critical limits of different eco-physiological factors affecting fungal infection and
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41 56 mycotoxin synthesis, but in many cases, the use of fungicides is the only efficient, cost-
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43
44 57 effective, and often successful way to prevent the mould growth (Munimbazi *et al.*
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46 58 1997).
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49
50 60 The aim of this study was to evaluate the impact of the application of several fungicides
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53 61 to grapes, on *A. carbonarius* growth and OTA production. The experimental design was
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55 62 divided in three parts in order to: i) screen the main fungicides used in Spanish vines, to
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58 63 test their efficiency against *A. carbonarius* growth and OTA production on synthetic
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60 64 nutrient medium (SNM); ii) find out the minimum inhibitory concentration (MIC) of

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3 65 each fungicide for *A. carbonarius* growth on SNM; iii) investigate the effect of several
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5 66 fungicides on growth and OTA production of *A. carbonarius* inoculated on grapes.
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9
10 68 **Material and methods**

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12 69 *Screening of the main fungicides used in vines on synthetic grape-like medium*

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15 70 Three ochratoxigenic *A. carbonarius* strains (3.161, 3.162 and 3.168, grape-isolated
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17 71 from Italy, France and Spain, respectively), were used to centrally inoculate (10^6 spores
18
19 72 ml^{-1}) Petri dishes containing 20 ml of synthetic nutrient medium (SNM), which had a
20
21 73 composition similar to grapes, and a water activity (a_w) level of 0.99 a_w (Bellí et al.
22
23 74 2004b). Strains are held in the culture collection of the Food Technology Department,
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25 75 University of Lleida, Spain.
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31
32 77 Twenty-six fungicides commonly used on Spanish vines, were added to the medium at
33
34 78 the doses recommended by manufacturers (Table 1). Each fungicide was aseptically
35
36 79 added to the autoclaved medium before plating it. No fungicides were added to control
37
38 80 plates. Plates were incubated at 20 °C and 30 °C inside plastic bags. Colony diameters
39
40 81 were measured after 3, 5 and 7 days and OTA was extracted after 7 days following the
41
42 82 method of Bragulat *et al.* (2001).
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47 83 High performance liquid chromatography (HPLC) with fluorescence detection (Waters
48
49 84 474, Milford, Massachusetts, U.S.A.) (λ_{exc} 330 nm; λ_{em} 460 nm) was used for OTA
50
51 85 analysis. The mobile phase was acetonitrile-water-acetic acid (57:41:2) (1.0 ml min^{-1})
52
53 86 and a C_{18} column (Waters Spherisorb 5 μm , ODS2, 4.6x250 mm) was used. The
54
55 87 injection volume and the retention time were 25 μl and 7.1 min, respectively. The
56
57 88 detection limit of the analysis was 0.02 $\mu\text{g OTA g}^{-1}$ of SNM, based on a signal-to-noise
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3 89 ratio of 3:1. The ochratoxin A standard was from *A. ochraceus* (Sigma-Aldrich,
4
5 90 Steinheim, Germany). The standard solution was made in methanol and concentration
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8 91 confirmed by using an UV spectrophotometer. Three repetitions were carried out, both
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10 92 for growth and OTA studies.
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15 94 [Insert table 1 about here]
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20 96 *Minimum doses preventing A. carbonarius growth on synthetic grape-like medium*
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22 97 All the fungicides that prevented *A. carbonarius* growth at the doses recommended by
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24
25 98 the manufacturers in the previous experiment (n=13) were selected for this study in
26
27 99 order to find the minimum concentration that inhibited the growth of this mould (MIC).
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29
30 100 Spore suspensions of 3.162 and 3.168 *A. carbonarius* strains were adjusted to contain
31
32 101 approximately 10^6 spores ml^{-1} for use as inoculum. SNM plates (0.99 a_w) with
33
34 102 decreasing concentrations of those fungicides (D, dose recommended by the
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36
37 103 manufacturer; d1, 0.75xD; d2, 0.5xD; d3, 0.25xD; d4, 0.1xD; d5, 0.01xD and d6,
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39 104 0.005xD) were single-point inoculated and incubated at 20 °C and 30 °C. Growth was
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41 105 measured daily during a period of 30 days. Three repetitions were carried out.
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46 107 *Effect of fungicides on grapes*
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48 108 The effect of six fungicides applied directly to grapes was investigated. F3, F14 and F26
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51 109 were used at the dose recommended by the manufacturer and F1, F9 and F23 were used
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53 110 at d4 (0.1xD). Table grapes (Red Globe var.) were surface disinfected by dipping them
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55
56 111 in NaClO (0.1 % Cl) and ethanol (70 %) solutions for 30 seconds and excess of
57
58 112 moisture was aseptically removed. Afterwards, 20 grapes were dipped in a fungicide
59
60 113 solution for 30 seconds and were placed onto a grid inside plastic boxes containing 300

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2
3 114 ml of water to keep a high relative humidity (90-100%) throughout the experiment. In
4
5 115 control treatments, grapes were dipped in water instead of fungicide. For culturing, 10^3
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8 116 spore ml^{-1} suspensions of two *A. carbonarius* strains (3.162 and 3.168), were sprayed
9
10 117 onto the grapes. After 7 days of incubation at 20 °C and 30 °C, the percentage of grapes
11
12 118 infected by *A. carbonarius* was assessed. The whole set of grapes of each treatment
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14
15 119 were crushed, and filtered through Whatman n°1 filter paper under vacuum. OTA was
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17 120 extracted from this must following the method of Bezzo *et al.* (2000). Twenty-five μl of
18
19 121 each sample were injected into the HPLC system equipped with a fluorescence detector
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21 122 (Waters 474, Milford, Massachusetts, U.S.A.) (λ_{exc} 230 nm; λ_{em} 458 nm) and a C_{18}
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23 123 column (Waters Spherisorb 5 μm , ODS2, 4.6x250 mm). The analysis was performed
24
25 124 under isocratic conditions, with acetonitrile 48% -sodium acetate 4mM/acetic acid
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27 125 (19/1)- 52%, as the mobile phase, pumped at a flow rate of 1 ml min^{-1} . The injection
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29 126 volume and the retention time were 25 μl and 12 min, respectively. The detection limit
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31 127 of the analysis was 0.05 $\mu\text{g l}^{-1}$, based on a signal: noise ratio of 3:1. OTA was quantified
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33 128 by the external standard method. The ochratoxin standard was from *Aspergillus*
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35 129 *ochraceus* (Sigma-Aldrich, Steinheim, Germany). The standard solution was made in
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37 130 methanol and confirmed by using an UV spectrophotometer.
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46 132 *Statistical treatment of the results*

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49 133 The regression lines of colony diameters against days after inoculation were calculated
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51 134 for each fungicide and were used to obtain the growth rate under each treatment
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53 135 conditions. Fungicide effect on mycelial growth on SNM and OTA production, both in
54
55 136 medium and in natural grapes, were statistically analysed with SAS Enterprise Guide
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57 137 software (SAS Institute, version 2.0, Inc., Cary, N.C., U.S.A.) by analysis of variance
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3 138 followed by either LSMEAN or Duncan multiple range tests. Statistical significance
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5 139 was judged at $P < 0.001$.

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10 141 **Results**

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12 142 *Screening of the main fungicides used in vines on synthetic grape-like medium*

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15 143 Significant differences were detected for the single factors temperature and fungicide
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17 144 and their interaction, while all the strains showed statistically similar growth, regardless
18
19 145 of the assayed levels of the remaining factors. Growth was faster at 30 °C than at 20 °C,
20
21 146 except for F2 and F11. Nine fungicides significantly reduced *A. carbonarius* growth
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23 147 rate at both temperatures (F2, F5, F7, F11, F13, F14, F20, F21 and F26) in comparison
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25 148 with the control treatment, meanwhile thirteen fungicides completely inhibited fungal
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27 149 growth at the dose assayed (F1, F4, F6, F9, F10, F12, F15, F16, F18, F19, F23, F24 and
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29 150 F25) (Table 2). No significant effects were observed for the remaining four fungicides
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31 151 on growth.

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39 153 The fungicides had a significant effect on OTA production by *A. carbonarius*, because
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41 154 obviously the 13 fungicides that prevented fungal growth also inhibited OTA
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43 155 production. Among the remaining fungicides, analysis of variance showed that none of
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45 156 them reduced significantly OTA production. No significant differences were found
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47 157 among the isolates tested, either in their response to temperature and fungicide
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49 158 treatments. The interaction fungicide x temperature was also significant. Contrary to the
50
51 159 growth pattern, OTA production was in general higher at 20 °C than at 30 °C. Although
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53 160 not having a significant weight, general trends can be drawn from the results. Mean
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55 161 levels of OTA production showed that most of the fungicides that reduced *A.*
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57 162 *carbonarius* growth, also reduced OTA production, with the exception of F2, F5 and

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3 163 F11, which favoured toxin production at both temperatures. OTA was also favoured by
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5 164 the addition of F7 at 20 °C and F14, F21 and F26 at 30 °C. OTA was also higher than
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8 165 the control under the effect of F8 at both temperatures although growth was only
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10 166 stimulated at 30 °C. Contrary, F17 reduced OTA production although growth was
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12 167 favoured at 20 °C. OTA was favoured at 20 °C and 30 °C under the effect of F22, the
13
14 168 unique fungicide that increased fungal growth at both temperatures. Figure 1 compares
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16 169 the growth rate and the amount of OTA detected at both temperatures after the
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18 170 application of each fungicide with the control treatment, which is represented at the
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20 171 origin of coordinates. Control growth and OTA production detected after the application
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22 172 of each fungicide has been subtracted from growth and OTA production by the control
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24 173 treatment. Thus, fungicides in the third quadrant of the graphic resulted in a reduction of
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26 174 both growth and OTA production.
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34 176 [Insert table 3 and figure 1 about here]

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37 178 *Minima doses preventing A. carbonarius growth on synthetic grape-like medium*

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39 179 No significant differences were found between the two strains of *A. carbonarius* tested
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41 180 (data not shown). At the optimum temperature for *A. carbonarius* growth, 30 °C, higher
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43 181 concentration of fungicide was needed to prevent fungal growth than at 20 °C. Each
44
45 182 fungicide had a different effect on *A. carbonarius* growth, but most of them were
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47 183 effective at doses around 1/4th (d3) of the dose recommended by the manufacturer (D)
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49 184 (Table 3). Only three fungicides did not prevent growth at this dose: F4 at D (d1) and
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51 185 F1 and F16 at 0.50 x D (d2), in the assays at 30 °C. 1/10th of D (d4), was the MIC of
52
53 186 fungicides F4 and captan 50% (F18) at 20 °C, and fludioxonil (F6) and tolyfluanid
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55 187 (F15) at both temperatures. A mixture of cyprodinil 37.5% and fludioxonil 25% (F9)

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3 188 and cyprodinil alone (F10), were the most effective fungicides, as they hinder growth at
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5 189 the minimum doses assayed (d5 and d6, respectively).
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10 191 [Insert table 3 about here]
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15 193 *Effect of fungicides on grapes*
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17 194 Three fungicides that completely inhibited *A. carbonarius* on SNM: penconazole 10%
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19 195 (F1), cyprodinil 37.5% + fludioxonil 25% (F9) and mancozeb 80% (F23), and two
20
21 196 fungicides that reduced its growth at the initial doses assayed: fenhexamid 50% (F14)
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23 197 and copper hydroxide 80%+copper 50% (F26), plus azoxystrobin 25% (F3) were
24
25 198 chosen for this study. The factors fungicide and temperature were significant in both
26
27 199 grape infection and OTA production experiments, and the factor strain only in the OTA
28
29 200 production trial (data not shown). The percentage of grapes infected by *A. carbonarius*
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31 201 was calculated for each treatment. Infection was significantly higher at 30 °C than at 20
32
33 202 °C. A percentage of reduction of the percentage of infection for each treatment was
34
35 203 determined by comparison with the control. The average of the percentage of reduction
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37 204 of both strains is shown in Figure 2. Infection was reduced at both temperatures with
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39 205 azoxystrobin 25% (F3) and cyprodinil 37.5%+fludioxonil 25% (F9), and with
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41 206 penconazole 10% (F1) at 20 °C. Maximum reduction (> 95 %) was achieved with
42
43 207 cyprodinil 37.5%+fludioxonil 25% (F9) at 20 °C, followed by azoxystrobin 25% (F3)
44
45 208 (65 %) and penconazole 10% (F1) (59 %) at the same temperature. Around 10 %
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47 209 reduction in the infection percentage was also detected with mancozeb 80% (F23) at 20
48
49 210 °C. In general, fenhexamid 50% (F14), mancozeb 80% (F23) and copper hydroxide
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51 211 80%+copper 50% (F26) enhanced *A. carbonarius* grape infection, especially at 30 °C.
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215 Half of the fungicides (penconazole 10%, azoxystrobin 25% and cyprodinil
216 37.5%+fludioxonil 25%; F1, F3 and F9) showed a clear reduction of the OTA
217 production at both temperatures, in comparison with the control treatment (Figure 3).
218 For these fungicides, reduction was higher at 20 °C than at 30 °C, except for
219 penconazole 10% (F1) for 3.162 strain. Differences in the percentage of reduction were
220 also observed between both strains, especially at 30 °C, where fungicides were more
221 effective against strain 3.168. At 20 °C, fenhexamid 50% (F14), mancozeb 80% (F23)
222 and copper hydroxide 80%+copper 50% (F26) reduced the OTA production for strain
223 3.168 meanwhile they produced the opposite effect on strain 3.162, increasing OTA
224 production more than 200 % sometimes. At 30 °C, these three fungicides increased up
225 to 20 % OTA production of both strains.

226
227 [Insert figure 3 about here]
228

229 Discussion

230 A significant effort has been concentrated on the development and use of fungicides for
231 the control of other food-spoilage fungi like *Fusarium* spp. (Mathies and Buchenauer
232 1996, Moss and Frank 1985), *Aspergillus flavus* and *A. ochraceus* (Munimbazi *et al.*
233 1997), *Botrytis cinerea* (Slawecki *et al.* 2002), etc, but not any studies on grapes.
234 Existing earlier studies have shown that combinations of Euparen (a sulfamide type
235 fungicide) and Mycodifol (Karadimcheva 1978), or captan (Tandon *et al.* 1975) were
236 found to be effective against black aspergilli colonising grape berries. Data on
237 resistance of *A. carbonarius* to fungicide treatments is non-existent so far. Therefore,

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3 238 the efficiency against this mould of a range of fungicides designed to control other
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5 239 species infecting vines, was tested in this study. Moreover, it has to be underlined, that
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8 240 the doses proposed by the manufacturers and used in the present study, were also
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10 241 designed for the control of other moulds.
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15 243 To discuss the results obtained, the different fungicides may be grouped according to
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17 244 their active ingredients. *A. carbonarius* growth and OTA production were minimised
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19 245 when using fungicides with copper in their composition (F20, F21, F24, F25 and F26).
20
21 246 However, copper oxychloride was also present in F5, which although limiting *A.*
22
23 247 *carbonarius* growth, did not decrease OTA production. The same OTA-enhancing
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25 248 effect was detected for fluazinam (F2) and procymidone (F11), which were classified as
26
27 249 dinitro aniline and dicarboximide fungicides, respectively. It is known that fluazinam
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29 250 (F2), together with azoxystrobin (F3, F13 and F17), thifluzamide and carboxin can
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31 251 interfere with respiration processes (Corbett *et al.* 1984, Guo *et al.* 1991, Sauter *et al.*
32
33 252 1995). Both *A. carbonarius* growth and OTA production were increased, although not
34
35 253 significantly, when adding inorganic fungicides containing sulfur to the medium (F8
36
37 254 and F22). Similar effects were detected in a study of the OTA content in red wines
38
39 255 produced from vineyards treated with different pesticides (Lo Curto *et al.* 2004). The
40
41 256 level of OTA in wines from sulfur-treated grapes was higher than in the other samples.
42
43 257 Furthermore, those authors reported azoxystrobin as a fungicide able to reduce OTA
44
45 258 concentration in wine, with 96.5 % of reduction. Data on OTA concentration in wine
46
47 259 are not directly comparable to the screening in this study, because OTA concentration in
48
49 260 wine or grape accounts for total OTA. In our study, both OTA producing capacity must
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51 261 be coupled to colony size data to have an idea of the total OTA accumulation. Thus, in
52
53 262 the present study, the total amount of OTA produced by *A. carbonarius* growing with
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3 263 sulfur fungicides increased due to both the higher OTA-producing capacity detected and
4
5 264 the bigger diameters of the colonies. However, it is not clear whether the total amount
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7
8 265 of OTA accumulated by *A. carbonarius* treated with fluazinam (F2) and procymidone
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10 266 (F11) increased or not because OTA production stimulation occurred but, in contrast,
11
12 267 smaller colonies were observed. Some othe fungicides have been found to stimulate
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14
15 268 OTA production in grapes (Battilani et al. 2003).
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17 269
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19 270 Little is known about the mechanism of action of the active ingredients of the
20
21 271 fungicides assayed. For many compounds, spore germination is the growth stage that is
22
23 272 most sensitive to inhibition (Slawecki *et al.* 2002). In the present study, fungicides that
24
25 273 completely inhibited germination were enclosed in several groups according to their
26
27 274 active ingredients: amide (F12 –one component-, F15) and dicarboximide fungicides
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29 275 (F12 –one component-, F18), triazol fungicides (F1 and F16), benzimidazole (F19) and
30
31 276 dithiocarbamate fungicides (F23, F24 and F25 –one component-), pyrimidine fungicides
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33 277 (F9 -one component- and F10), phenylpyrrole fungicides (F6 and F9 –one component-),
34
35 278 etc. It would be interesting to study growth for longer periods in order to know if this
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37 279 last group of fungicides at the doses assayed, totally inhibited growth or only prolonged
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39 280 the lag phase of the mould. Doses of these fungicides were reduced in a subsequent
40
41 281 experiment in order to find the threshold dose preventing *A. carbonarius* growth. In
42
43 282 general, fungicides with the same active ingredients seemed to have similar effects
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45 283 when reducing the doses. Triazol fungicides (F1 and F16) were the less effective
46
47 284 fungicides against *A. carbonarius* growth, as just reducing up to one quarter the initial
48
49 285 dose the inhibitory growth effect disappeared. Fungicides with the MIC at one quarter
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51 286 of the initial one (F19, F23, F24 and F25), were classified as carbamate and
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53 287 dithiocarbamate fungicides. Cyprodinil seemed to be the active ingredient more suitable
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3 288 to stop fungal growth, as it was a component of the pyrimidine fungicides F9 (Switch)
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5 289 and F10 (Chorus), which showed the minimum threshold concentrations. Oppositely,
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8 290 Greek authors observed that pesticides such as Carbendazim and Chorus were
9
10 291 ineffective in controlling sour rot caused by aspergilli (Tjamos et al. 2004). However,
11
12 292 the application of Switch led to significant decrease in incidence of black aspergilli on
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14
15 293 grapes. The fungicide Switch contains cyprodinil and fludioxonil which belong to the
16
17 294 pyrimidine and pyrrolnitrin classes of fungicides, respectively. Since the fungicide
18
19 295 Chorus contains cyprodinil and was ineffective against aspergilli, it was concluded that
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21 296 fludioxonil was the active ingredient of Switch (Tjamos et al. 2004).
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26
27 298 Sauter *et al.* (1995) noted that the group of fungicides containing the strobilurins,
28
29 299 blocked electron transport at the cytochrome *bc₁* complex of the mitochondrial electron
30
31 300 transport chain, and therefore were extremely potent inhibitors of spore germination, but
32
33 301 much less active as inhibitors of mycelial growth. No germination-inhibitory effect of
34
35 302 fungicides grouped as strobilurin fungicides (F3, F13 and F17) was noticeable in this
36
37 303 study, as the three of them allowed *A. carbonarius* growth, although less than the
38
39 304 control treatment. Other reported fungicides that typically acted after germination in
40
41 305 filamentous fungi by strongly inhibiting mycelial growth, included antimicrotubule
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43 306 agents (carbendazim and N-phenylcarbamates, which inhibited nuclear division (Suzuki
44
45 307 *et al.* 1984)), and inhibitors of ergosterol biosynthesis (Buchenauer, 1987). However, in
46
47 308 this study, benzimidazol (F19) and dithiocarbamate fungicides (F23, F24 and F25 –one
48
49 309 component-) showed a completely inhibition of germination as mentioned before.
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57 311 On grapes, a mixture of cyprodinil (37.5 %) and fludioxonil (25 %) (F9) seemed the
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59 312 best fungicide that controlled *A. carbonarius* growth and OTA production together with
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3 313 penconazole 10% (F1) and azoxystrobin 25% (F3). All three were also restrictive
4
5 314 fungicides in terms of growth and mycotoxin production when tested on SNM medium.
6
7 315 Penconazole, was previously reported as a synthetic pesticide able to reduce around 90
8
9 316 % the level of OTA in wines made from grapes treated with this fungicide (Lo Curto *et*
10
11 317 *al.* 2004). In another study carried out at the ITV France by Molot and Solanet (2003),
12
13 318 the fungicides Switch (F9), Scala (containing the pyrimidine fungicide pyrimethanil)
14
15 319 and Mikal (containing fosetyl-Al and the dicarboximide folpel) were found to be the
16
17 320 most effective for lowering fungal colonization and OTA content of wines Fenhexamid
18
19 321 50% (F14) and copper 50% (F26) showed the same effects on synthetic nutrient
20
21 322 medium than on grapes, as they increased OTA production, especially at 30 °C.
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23 323 Temperature was a determinant factor and could influence in the effectiveness of the
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25 324 fungicides. Results are in accordance with previous work, reporting optimum
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27 325 temperatures for *A. carbonarius* growth and OTA production at 30 °C and 20 °C,
28
29 326 respectively (Bellí *et al.* 2005). Propitious levels of other environmental factors, such as
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31 327 humidity, could also interfere in the efficacy of the fungicides assayed, together with the
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33 328 reiterative application of the same fungicide, as it could modify the equilibrium in the
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35 329 ecosystem, enhancing other microorganisms development, as competing fungi are
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37 330 removed.
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41 332 Additional *in vitro* studies on grapes testing the whole range of fungicides are needed in
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43 333 order to find out the best active ingredients against *A. carbonarius* development and
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45 334 mycotoxin production. Afterwards, further studies of the *in situ* efficiency of pesticide
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47 335 treatments against *A. carbonarius* infection and OTA production in vines would be
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49 336 required.
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4

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14
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438 ochratoxin A production and chemical control. *Journal of Phytopathology*, **152**, 250-
439 255.

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3 440 Table 1. Fungicides used in the study, their composition, origin and doses recommended
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5 441 by the manufacturers.
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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	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

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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444 Table 2. Mean growth rates (mm day⁻¹) and OTA production on Synthetic Nutrient
 445 Medium ($\mu\text{g g}^{-1}$ SNM) at two temperatures (20 °C and 30 °C) by three strains of *A.*
 446 *carbonarius* (3.161, 3.162 and 3.168). Values are the mean of the three strains and three
 447 replicates of each \pm standard deviation. Data in each column followed by different
 448 letters are significantly different in the Duncan test.
 449

Fungicide	Growth rate (mm day ⁻¹)		OTA production ($\mu\text{g g}^{-1}$ SNM)	
	20 °C	30 °C	20 °C	30 °C
Control	5.46 \pm 0.16 ^{ab}	7.98 \pm 0.32 ^{ab}	5.68 \pm 5.13 ^{bc}	1.14 \pm 1.17 ^c
F1	N.G.	N.G.	<d.l. ^d	<d.l. ^d
F2	1.73 \pm 0.15 ^{ed}	1.67 \pm 0.52 ^{fg}	19.66 \pm 16.24 ^a	14.20 \pm 0.81 ^b
F3	4.50 \pm 0.32 ^{bc}	7.79 \pm 1.77 ^{ab}	2.84 \pm 4.70 ^{bc}	0.22 \pm 0.39 ^c
F4	N.G.	N.G.	<d.l. ^d	<d.l. ^d
F5	1.53 \pm 0.63 ^{ed}	3.00 \pm 0.43 ^{ef}	13.34 \pm 20.29 ^{abc}	2.58 \pm 3.01 ^c
F6	N.G.	N.G.	<d.l. ^d	<d.l. ^d
F7	3.91 \pm 0.35 ^c	6.77 \pm 0.10 ^{bc}	14.41 \pm 17.98 ^{ab}	0.64 \pm 0.59 ^c
F8	5.24 \pm 0.65 ^{ab}	8.65 \pm 0.99 ^a	8.03 \pm 7.00 ^{abc}	8.92 \pm 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 \pm 1.56 ^d	1.59 \pm 0.18 ^g	11.29 \pm 12.74 ^{abc}	58.61 \pm 18.23 ^a
F12	N.G.	N.G.	<d.l. ^d	<d.l. ^d
F13	1.91 \pm 0.42 ^{ed}	2.66 \pm 0.22 ^{efg}	0.96 \pm 1.56 ^c	0.34 \pm 0.59 ^c
F14	3.92 \pm 0.29 ^c	6.34 \pm 0.43 ^c	4.28 \pm 3.73 ^{bc}	1.96 \pm 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

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^{cd}	1.97 ± 2.52^{bc}	1.86 ± 3.22^c

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

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

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

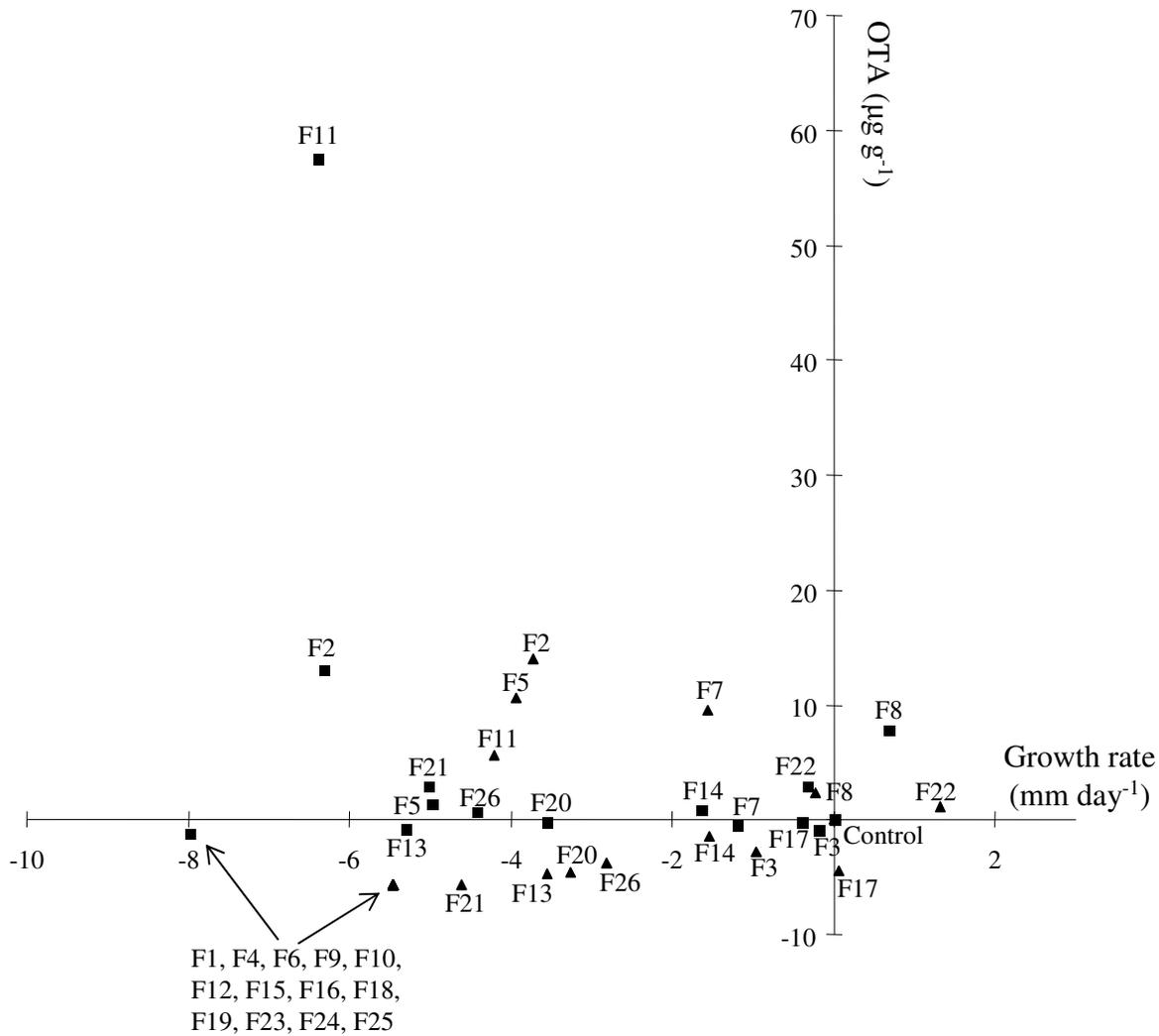
455 + *A. carbonarius* growth; – no growth.

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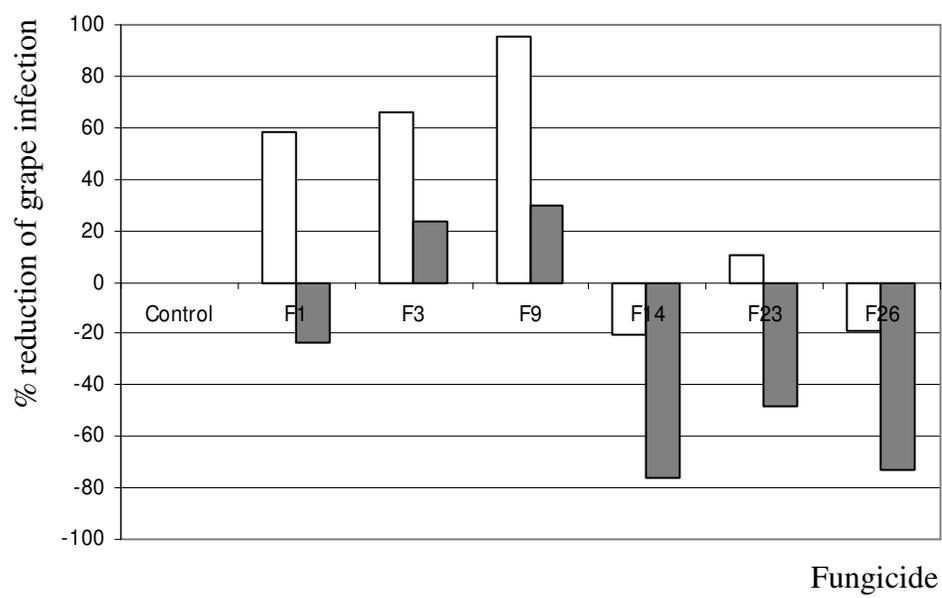
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3 457 Figure 1. Growth rate (mm day^{-1}) and OTA production ($\mu\text{g g}^{-1}$) at $\blacktriangle 20\text{ }^{\circ}\text{C}$ and $\blacksquare 30\text{ }^{\circ}\text{C}$
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5 458 of three strains of *A. carbonarius* (3.161, 3.162 and 3.168) after the addition of several
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7 459 fungicides (F1-F26) to the SNM medium. Values are the mean of the three strains. No
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9 460 fungicide was added to the control treatment and growth and OTA production was
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11 461 considered as zero.
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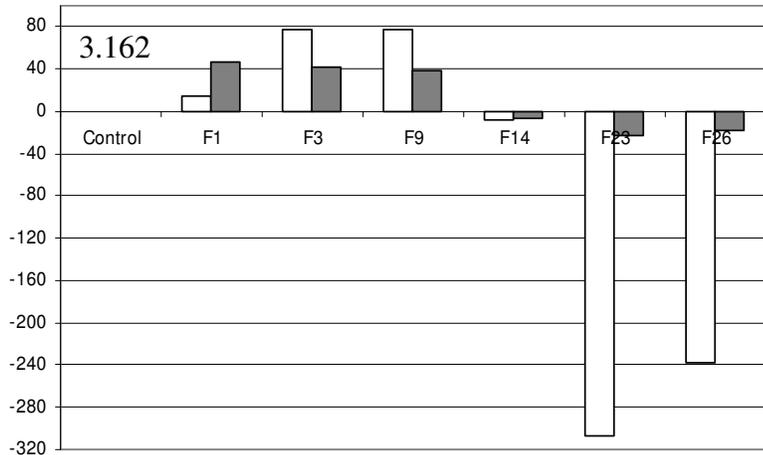
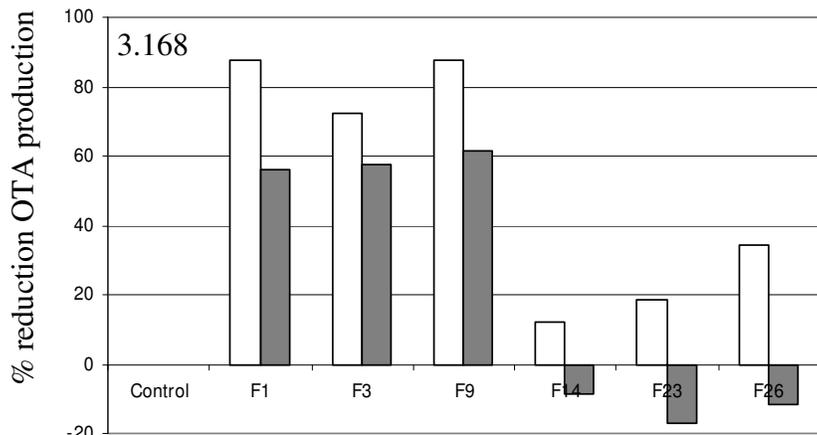
22 465 Figure 2. Percentage of reduction in the percentage of grape infection by *A. carbonarius*
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24 466 treated with different fungicides and incubated at $\square 20\text{ }^{\circ}\text{C}$ and $\blacksquare 30\text{ }^{\circ}\text{C}$. Values are the
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26 467 mean of two strains.
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35 471 Figure 3. Percentage of reduction of OTA production by two *A. carbonarius* strains
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37 472 (3.162 and 3.168) inoculated on grapes ($\mu\text{g ml}^{-1}$ must) treated with different fungicides
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39 473 and incubated at $\square 20\text{ }^{\circ}\text{C}$ and $\blacksquare 30\text{ }^{\circ}\text{C}$.
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